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13. ABSTRACT *(Maximum 200 Words)*

Purpose: This study was designed to identify one or more prostate cancer (CaP) tumor suppressor genes (TSG) on chromosome 8 by assessing the distribution of loss of heterozygosity (LOH) in brothers from multiplex sibships A strong linkage signal has been demonstrated in these families. LOH was assessed in 202 men with histologically confirmed prostate cancer. The differential pattern of linkage disequilibrium between affected men and controls was used to select 2 candidate genes on chromosome 8p (BNIP3L and CLUSTERIN) for SNP genotyping in 633 men with prostate cancer from 293 multiplex families and in 529 unrelated control subjects.
Major Findings: We confirm a high rate of LOH on chromosome 8, with significantly higher rates on 8p than 8q. No difference in allele frequency between cases and controls could be demonstrated for any of the 5 SNPs or their extended haplotypes genotyped in either of the 2 candidate susceptibility loci.

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FINAL PROGRESS REPORT

INTRODUCTION: The purpose of this study was to conduct a search for a tumor suppressor gene (TSG) or genes on chromosome 8 that, when inactivated, increase a man's susceptibility to develop prostate cancer (CaP). Our approach was to identify regions on chromosome 8 that display a similar pattern of loss of heterozygosity (LOH) in brothers with CaP. Based in part on the distribution of LOH determined in this study, two candidate TSGs were selected from a region of high LOH for further scrutiny. The results of the LOH analysis and the follow-up study of the 2 candidate genes are detailed in this final Progress Report.

BODY: Table 1 reports the distribution of LOH for 10 chromosome 8p and 4 chromosome 8q microsatellites genotyped in this study. This is the same table as was reported in the last progress report, except that the sample size for 6 markers (D8S262, D8S277, D8S351, D8S560, D8S1822 and D8S521) has been increased by an additional 788 genotypes. Significantly more LOH was observed for the microsatellite markers genotyped on 8p compared to the microsatellite markers genotyped on 8q (38.2% vs. 26.6%, chi-square = 6.2, $p<0.02$).

Table 1

Marker	Percent Heterozygosity	Percent Informative with LOH
D8S1781	87.5	0.0
D8S262	67.8	32.8
D8S277	78.1	47.4
D8S351	89.3	31.5
D8S1825	66.2	46.8
D8S1130	84.1	33.3
D8S1106	74.2	33.7
D8S1731	85.6	36.8
D8S261	68.6	45.8
D8S560	72.7	44.6
centromere		
D8S1144	68.2	26.7
D8S175	62.9	27.3
D8S1822	85.1	35.1
D8S521	75.3	15.5

Every LOH study of prostate cancer with which we are familiar has been carried out in panels of unrelated men. Our idea was to undertake an LOH study of brothers from sibships that contained at least 3 affected members and to use the power of the family study design to help

pinpoint the location of TSGs. We chose to concentrate on two regions of chromosome 8 because linkage analysis of these trio families indicated that our sample was likely segregating a TSG in one or both of these regions. Our original hypothesis remains the same, namely that the pattern of LOH (with respect to which allele or haplotype is retained) will be correlated within sibships as a function of the sibship's identity-by-descent (IBD) configuration. This hypothesis logically follows from (and tests the assumption that) the excess allele sharing revealed by our multipoint linkage analysis is due to the presence of a shared "mutated" tumor suppressor gene in a proportion of these sibships.

For reasons given in the last progress report, we were unsuccessful in obtaining archival samples for many of the men used in the original linkage analysis. Either the samples do not exist, could not be located, or consisted only of a needle biopsy. During the extension year, we were able to obtain paraffin blocks on an additional 38 subjects, bringing the total to 195 paraffin blocks. While this number is larger (by about 56%) than the number we proposed to genotype in our original DOD application, the actual number of samples that were obtained from all 3 members of a brother trio remains small.

Table 2 reports the distribution of the 6 possible LOH patterns observed to date in affected brothers. Table 2 is based on only 410 genotypes--a sample size too small to rigorously test the hypothesis.

Table 2

Pattern	Percent
Homozygous/Homozygous	12.7
Homozygous/No LOH	17.6
Homozygous/LOH	5.9
No LOH/No LOH	34.1
No LOH/LOH	23.4
LOH/LOH	6.3

When the paraffin blocks were received, they were sectioned, mounted and stained. The sections were examined by a pathologist and those sections that contained sufficient tumor were sent to colleagues in Pittsburgh or Denver who prepared the laser capture microdissected (LCMD) caps. Our experience is that the amount and quality of tumor DNA varied widely from cap-to-cap. Under the best conditions, our genotyping laboratory was able to perform 8 PCR reactions per cap although the modal number was far fewer.

Accordingly, during the one-year no-cost extension of this study we decided that we needed some way of increasing the supply of tumor DNA. To this end we established a collaboration with a molecular genetics laboratory in Cincinnati with expertise in performing whole genome amplification (WGA) of minute archival DNA samples to determine if WGA could be used on our LCMD caps to increase the yield of tumor DNA. While this laboratory had no previous experience with tumor DNA (they mainly perform WGA on forensic DNA samples from crime scenes and occasionally archaeological samples), a pilot test on a LCMD cap suggested that WGA would be a feasible strategy. The cost of this work was entirely supported by a private research foundation.

We had on hand 157 duplicate LCMD caps at the beginning of the extension year. These and the corresponding constitutional DNAs were sent to the Cincinnati lab for WGA. As mentioned above, LCMD caps from an additional 38 subjects were obtained during the extension year and they and their paired constitutional DNAs were also sent to Cincinnati for WGA. As of Sept. 26, 2002, 117 tumor DNA samples and 132 constitutional DNA samples have been subjected to WGA. All of the constitutional DNA samples were successfully amplified. However, WGA was successful for only 20 (17%) of the tumor samples. Our collaborators in Cincinnati believe that the low success rate is due to impurities that result from the process of staining the samples prior to microdissection. The lab has developed a new assay for stained samples that is currently being evaluated. We have been assured that there is sufficient tumor DNA on hand that if the new assay is successful, all of the samples should be amplifiable.

CANDIDATE GENES: The ultimate goal of this study was to identify one or more TSGs. Based in part on the distribution of LOH shown in Table 1, the project's molecular geneticist, Dr. D. S. Gerhard, selected two candidate genes closest to the marker that (at the time of their selection) showed the greatest LOH (D8S560). The two genes are BNIP3L (NT_023666), a homolog of the previously identified proapoptotic protein BNIP3, and CLUSTERIN (NT_007988), also known as Testosterone-Repressed Prostate Message 2, that encodes a protein that is believed to be involved in the cascade of events leading to programmed cell death. Five SNPs were genotyped in these two genes. In this report we will refer to the 5 SNPs as SNP1, SNP2, SNP3 (in and around BNIP3L), and SNP4 and SNP5 (in the CLUSTERIN gene). Table 3 reports the particulars for these SNPs.

Table 3

Designation	rs Number	Contig Position	Type, Location of SNP
SNP1	2874670	3233478	C/T transition, intronic
SNP2	2304300	3236841	A/G transition, intronic
SNP3	12165	3257237	A/G transition, 3' UTR
SNP4	1049263*	318482	C/T transition, exonic
SNP5	2279590	312254	C/T transition, intronic

*synonymous with rs7982

These candidate SNPs were genotyped in 633 men with prostate cancer who are members of 293 multiplex sibships (Suarez et al., 2000a,b; 2001) and in a large series of control subjects. The control subjects were ascertained from a large pool of men who have been followed for many years as part of a long-term prostate cancer screening study in which the subjects are screened at 6 to 12 month intervals with prostate specific antigen (PSA) blood tests and digital rectal examination (DRE) of the prostate (Smith et al., 1997). Controls were required to: 1) be at least 65 years old, 2) never have registered a PSA level in excess of 2.5 ng/ml or have had a DRE suspicious of CaP and, 3) have no known family history of CaP. All subjects in this study are of European ancestry. The number of controls who were successfully genotyped ranged from a low of 523 (for SNP2) to 529 (for SNP4). A total of 6,345 SNP genotypes were produced in the process of characterizing these 2 candidate susceptibility loci.

STATISTICAL AND GENETIC ANALYSIS: Maximum likelihood allele frequency estimates for the CaP cases were obtained from the USERM13 subroutine of the MENDEL computer package (Lange et al., 1988; Boehnke, 1991). Gene frequency estimates for the control sample were obtained by direct gene counting and used to predict Hardy-Weinberg proportions, the significance of which were evaluated with a likelihood ratio chi-square on 1 degree of freedom (Sham, 1998). Equality of allele frequencies between cases and controls was assessed with a 2-sample proportion test (Fleiss, 1973). The results of these analyses are reported in Table 4.

Table 4

Designation	Major Allele Frequency Cases	Major Allele Frequency Controls	Case vs Control p-value	Control HWE p-value
SNP1	0.571	0.575	0.86	0.40
SNP2	0.640	0.632	0.70	0.24
SNP3	0.679	0.671	0.69	0.59
SNP4	0.578	0.601	0.25	0.25
SNP5	0.588	0.607	0.35	0.25

It is becoming increasingly clear from numerous SNP studies that the prior probability of identifying functional SNPs (or SNPs that are in linkage disequilibrium with functional susceptibility elements) is low. All 5 SNPs we genotyped are in Hardy-Weinberg equilibrium. Comparison of the BNIP3L and CLUSTERIN SNP frequencies between cases and control reveals no evidence that these genes are involved in the initiation or progression of prostate cancer.

Haplotype frequencies were estimated using the EM algorithm as implemented in the ASSOCIATE program (Ott, 1985). Linkage disequilibrium estimates were converted to their "normalized" D' values, as described elsewhere (Suarez et al., 1994). The results of these analyses are reported in Table 5. The D' values are given above the Table's diagonal, and the p-values are below the diagonal.

Table 5

	SNP1	SNP2	SNP3	SNP4	SNP5
SNP1	-	0.968	1.000	0.069	0.067
SNP2	<0.001	-	0.876	0.093	0.111
SNP3	<0.001	<0.001	-	0.116	0.088
SNP4	0.108	0.173	0.111	-	0.944
SNP5	0.124	0.108	0.227	<0.001	-

In retrospect, given the almost total linkage disequilibrium between the SNPs within each gene, we could have reduced our genotyping effort substantially had this been known in advance. BNIP3L and CLUSTERIN are about 1 megabase apart and there is no evidence of intergenic disequilibrium. As with the individual SNP frequencies, cases and controls do not differ in their haplotype frequencies.

Finally, for completeness, the evidence for linkage between CaP and each of the 5 SNPS was evaluated with the computer program GENEHUNTER-PLUS (Kruglyak et al., 1996; Kong and Cox, 1997) in the multiplex trio families that were originally responsible for focusing our attention on chromosome 8p. The multipoint NPL Z-scores range from 2.23 to 2.25, as expected for markers in families already known to show linkage.

KEY RESEARCH ACCOMPLISHMENT:

- Fourteen chromosome 8 microsatellites were genotyped in a total of 202 men from multiplex prostate cancer families.
- Significantly greater LOH was observed for chromosome 8p markers than for 8q markers.
- The percent of informative markers with LOH ranges from 0% for the telomeric microsatellite D8S1781 to 47.4% for D8S277.
- 34.1% of informative markers in pairs of brothers show no LOH, while 23.4% reveal LOH in one of the brothers but not the other. For 6.3% of the markers, both brothers show LOH.
- We have developed a new statistic that exploits differences in patterns of linkage disequilibrium between cases and controls that should help delimit the position of a TSG on chromosome 8.
- We have developed a method that maps the limits of purely epistatic models and are developing techniques capable of identifying participating susceptibility loci.
- Two candidate susceptibility genes on chromosome 8p were genotyped for 5 SNPs in 633 men with prostate cancer (from multiplex families) and 529 healthy controls. No differences in the frequency of any of the SNPs (or SNP haplotypes) could be demonstrated in this large sample.

REPORTABLE OUTCOMES:

The following manuscripts have been published or are under review. Copies of 1 through 4 were included with the last Progress Report. Copies of 5 through 8 are included with this final Progress Report.

1. Suarez BK, Gerhard DS, Lin J, Haberer B, Nguyen L, Kesterson NK, and Catalona WJ: Polymorphisms in the prostate cancer susceptibility gene HPC2/ELAC2 in multiplex families and healthy controls. Cancer Res. 61:4982-4984 (2001).
2. Culverhouse R, Lin J, Liu K-Y, and Suarez BK: Linkage disequilibrium mapping in population isolates. Genet. Epid. 21:S429-434 (2001).
3. Suarez BK, Lin J, Catalona WJ, Haberer B, and Gerhard DS: CAG and GGC trinucleotide repeats in the androgen receptor gene and prostate cancer: The long and the short of it. Under revision.
4. Culverhouse R, Suarez BK, Lin J, and Reich T: A perspective on epistasis: Limits of models displaying no main effect. Am. J. Hum. Genet. 70:461-471 (2002).
5. Suarez, BK, Lin J, Catalona WJ, Haberer B, Gerhard DS: Trinucleotide repeats in the androgen receptor gene are not related to the risk of prostate cancer. Poster presented at the Am. Urol. Assoc. annual meeting, Orlando, FL, 2002.

6. Reding DJ, Zhang KQ, Salzman SA, Thomalla JV, Riepe RE, Suarez BK, Catalona WJ, Burmester JK: Identification of a gene frequently mutated in prostate tumors. Medical Oncology, 18:179-187 (2001).
7. Zhang KQ, Salzman SA, Suarez BK, Catalona WJ, Burmester JK: Genetics of prostate cancer. Clin. Med. Res. (in press)
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CONCLUSIONS:

Prostate cancer is the most common malignancy and second leading cause of cancer-related death among American men. It is estimated that last year, 198,000 men were newly diagnosed and about 31,300 died of the disease (Greenlee et al., 2001). There is now a persuasive body of evidence implicating one or more tumor suppressor genes on chromosome 8. This evidence derives from many LOH studies of unrelated men with prostate cancer. Because these studies are difficult, most findings have been based on relatively small sample sizes. We have adopted a different strategy in this study, namely to investigate the pattern of LOH in related subjects from multiplex sibships. We believe this will be the first LOH study of related men with prostate cancer.

We have assembled and genotyped tumor and constitutional DNA from over 200 men who come from multiplex families. We confirm a high rate of LOH on chromosome 8--with significantly higher rates on 8p than 8q. Although this panel is perhaps the largest ever genotyped for an LOH study of prostate cancer, we are still in the process of locating tumor samples for subjects who are from sibships with 3 or more affected brothers. Additionally, we have instituted a collaboration that will use whole genome amplification on tumor DNA so as to provide us with sufficient samples for future studies.

The distribution of LOH observed in our material led us to select 2 candidate susceptibility genes for SNP genotyping. These genes are BNIP3L and CLUSTERIN, located about one megabase apart on 8p21-12. Five SNPs were genotyped in these 2 candidates in 633 prostate cancer cases and 529 controls. Unfortunately, no individual SNP, nor any SNP haplotype, distinguishes the cases from the controls.

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Prostate Cancer: Basic Research (I)

Unmoderated Poster

Sunday, May 26, 2002 7:00 AM-4:30 PM

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NONSTEROIDAL ANTIANDROGENS FAIL TO INHIBIT ANDROGEN RECEPTOR ACTIVITY INDUCED BY ONCOSTATIN M *Sonia Godoy-Tundidor*, Alfred R Hobisch, Karina Pfeil, Georg Bartsch, Zoran Culig, Innsbruck, Austria*

INTRODUCTION AND OBJECTIVES: Oncostatin M (OSM), a member of the interleukin-6 (IL-6) family of cytokines that is produced by activated monocytes, T-lymphocytes, and in testis regulates growth of prostate cancer cells in a paracrine manner. It was demonstrated that IL-6 modulates cellular events in prostate cancer by activation of the androgen receptor (AR). This study was designed to investigate regulation of AR signaling by OSM in carcinoma of the prostate.

METHODS: OSM receptor expression in prostate cancer cell lines was investigated by RT-PCR. Transient transfections with the androgen-inducible reporter gene ARE2TATA-CAT and AR cDNA were carried out in DU-145 cells. Transfected cells were treated with androgen and/or OSM, in the absence or presence of nonsteroidal antiandrogens or inhibitors of protein kinase pathways. In one series of experiments, the cells were cotransfected with a dominant-negative mutant of the signal transducer and activator of transcription (STAT) 3 which is activated by IL-6 and related cytokines. Reporter gene activity was determined by CAT assay. AR expression in transfected cells was assessed by Western blot.

RESULTS: The OSM receptor expression was demonstrated in DU-145 and PC-3 cells. The AR was activated by OSM in a ligand-independent, dose-dependent manner and maximal activation was 58% of that achieved by androgen. Low doses of androgen and OSM enhanced AR activity in an additive manner. In the presence of OSM, hydroxyflutamide behaved like an agonist increasing OSM-induced AR activity two-fold. Bicalutamide was not efficient in inhibition of reporter gene activity. The protein kinase A inhibitor PKI caused only a 10% reduction of AR activity and a 25% inhibition was achieved with the inhibitor of the mitogen-activated protein kinase pathway PD 98059. The inclusion of a dominant-negative STAT 3 did not abolish activation of the AR by OSM. Western blot experiments did not reveal any change in AR expression after OSM treatment in transfected DU-145 cells.

CONCLUSIONS: In contrast to ligand-independent activation of the AR by IL-6, up-regulation of AR activity by OSM cannot be down-regulated by nonsteroidal antiandrogens. This might be due to recruitment of different intermediary signaling proteins by the two related cytokines. AR activation by OSM might be, in part, relevant to failure of prostate cancer endocrine therapy.

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DEVELOPMENT OF AN ORTHOTOPIC IMMUNOCOMPETENT MOUSE MODEL OF PROSTATE ADENOCARCINOMA *Roy R Brown*, Kenneth D Somers, Daniel A Holterman, William F Glass, George L Wright, Paul F Schellhammer, Richard P Ciavarra, Norfolk, VA*

INTRODUCTION AND OBJECTIVES: We established a new orthotopic model of mouse prostate adenocarcinoma in order to generate reproducible primary and metastatic carcinoma in C57BL/6 mice. Additionally, we assessed the in vivo efficacy of FLT3 ligand (L) in the treatment of C57BL/6 mice with orthotopic, metastatic prostate carcinoma.

METHODS: **Cytokine:** FLT3-L was provided by Immunex Corporation, Seattle, WA, and diluted in carrier solution (0.1% normal mouse serum (NMS) in PBS). **Flow cytometry:** Primary tumors dissociated in collagenase buffer or derived cell lines were assessed by flow cytometry for DNA content by propidium iodide staining. **In vivo studies:** Mice were injected in the right posterior lobe of the prostate with 5x10e5 TRAMP-C1 cells (obtained from Dr. Norm Greenberg, Baylor College of Medicine, Houston, TX.) resulting in primary tumors in 57-76 days. When clinically palpable, primary prostate adenocarcinomas were removed and the cells from the prostate and nodal sites were cultured in vitro. Cultured tumor cells were recycled three times by intraprostatic injection resulting in the in vivo selection and establishment of the recycled cell line TRAMP-C1P3 which reproducibly formed highly anaplastic primary tumors with paraaortic lymph node and distant organ metastases after ~30 days, in 19/20 (95%) of mice. To assess the effect of FLT3-L treatment on prostate tumor growth, mice were injected orthotopically with TRAMP-C1P3. Seven days post-inoculation, mice were randomly assigned for 28 consecutive days of treatment with either carrier or FLT3-L (30μg per injection). Mice were sacrificed at days 14 and 28 of therapy for primary and metastatic tumor assessment and evaluated by histopathology and flow cytometry.

RESULTS: Cell lines derived from LN metastases were aneuploid with DNA indices (DI) of 1.33-1.66 relative to TRAMP-C1P3 with DI of 1.5. We determined that FLT3-L therapy for 28 days suppressed primary tumor growth and metastatic disease in 8 of 9 mice (88%), with 25% tumor-free >4 months after completion of therapy. In contrast, all carrier-treated mice had clinically detectable prostate tumors, LN metastases and were moribund at 29-35 days.

CONCLUSIONS: We have established a reproducible and clinically relevant orthotopic animal model of prostate cancer with application to a variety of therapeutic strategies. The data demonstrate that FLT3-L treatment suppressed orthotopic prostate tumor growth and LN metastasis.

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TRINUCLEOTIDE REPEATS IN THE ANDROGEN RECEPTOR GENE ARE NOT RELATED TO THE RISK OF PROSTATE CANCER *Brian K Suarez*, Jennifer Lin, William J Catalona, Beth Haberer, Daniela S Gerhard, St. Louis, MO*

INTRODUCTION AND OBJECTIVES: The androgen receptor (AR) is indispensable for maturation and maintenance of the prostate. It is encoded by a single copy X-linked gene. Two polymorphic trinucleotide repeats; $(CAG)_n$, resulting in a polyglutamine tract, and $(GGC)_n$, resulting in a polyglycine tract, are present in exon 1 and are widely believed to affect risk for the development of prostate cancer (CaP).

METHODS: To test this and other hypotheses, we genotyped these 2 polymorphisms in 287 multiplex CaP sibships and in 353 race-matched controls. The controls were ascertained from a large sample of men who have been followed for many years as part of a long-term CaP screening study. To qualify as a control, subjects were required to be at least 65 years old, never have registered a PSA level in excess of 2.5 ng/ml (on a minimum of 3 occasions over the last 3 years), never have had a digital rectal examination suspicious of CaP, and have no known family history of CaP.

RESULTS: We find no difference in the cumulative distribution of allele frequencies between cases and controls for either polymorphism. Allele size (short, medium, long) is unrelated to age-at-diagnosis in our sample. There is, however, evidence of an interaction between the size of these trinucleotide repeats and Gleason score, such that CaP cases with short repeats for both microsatellites tend to have Gleason scores lower than predicted. We find no evidence of linkage disequilibrium between these polymorphisms in our control sample. By contrast, unrelated CaP cases present moderate evidence of increased linkage disequilibrium that results in an excess of short-CAG/short-GGC bearing haplotypes. Nonparametric analysis, however, finds no evidence for linkage of the AR gene to the risk of developing prostate cancer.

CONCLUSIONS: Although we find no evidence for a global difference in the distribution of allele sizes at either the CAG or the GGC repeat polymorphisms of the androgen receptor gene in men from multiplex CaP sibships compared with healthy controls, randomized replication tests suggest that a subset of short-CAG/short-GGC haplotypes may increase risk for the development of a nonaggressive form of CaP characterized by low Gleason scores.

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NON-ANDROGEN RECEPTOR-MEDIATED ANTIANDROGEN ACTION: ACTIVATION OF MAP KINASE PATHWAY BY HYDROXYFLUTAMIDE IN PROSTATE CANCER DU145 CELLS *Yi-Fen Lee*, Wen-Jye Lin, Jiaoti Huang, Edward Messing, Shang-Yi Chiu, Chawnshang Chang, Rochester, NY*

INTRODUCTION AND OBJECTIVES: While hydroxyflutamide has been used as an antiandrogen to block androgen-stimulated prostate tumor growth, the antiandrogen withdrawal syndrome which antiandrogens stimulate prostate tumor growth still occurs in many patients treated with androgen ablation therapy. This has previously been explained by mutations in the androgen receptor (AR) and/or modulation from AR coregulators, so that HF becomes an AR agonist.

METHODS: Using immunoblot of phosph-ERK1/2, we detected the phosphorylation of MAP kinase in DU145 prostate cancer cells, pretreated with MAP kinase inhibitors, anti-EGFR antibodies, or cycloheximide and followed by hydroxyflutamide, EGF, and vehicle treatments. We measured the cell proliferation profile upon the treatments by trypan blue exclusion assay. Finally, we were able to study four prostate cancer patients undergoing androgen ablation therapy with flutamide and compared their phosph-ERK1/2 levels in prostate cancer biopsies before receiving hydroxyflutamide and after experiencing disease progression while taking hydroxyflutamide.

RESULTS: We found that hydroxyflutamide induced activation of Ras/MAP kinase pathway within 5-15 min in a human prostate cancer cell line, DU145, that lacks the AR. Cycloheximide failed to inhibit this activation, but both AG1478, an inhibitor of the EGF receptor (EGF-R) and an EGF-R neutralizatng antibody blocked this hydroxyflutamide-mediated activation of MAP kinase, suggesting the activation of Ras/MAP kinase by HF is a membrane-initiated, non-AR mediated and non-genomic action. The consequence of this activation results in increased

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Original Article

Identification of a Gene Frequently Mutated in Prostate Tumors

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Abstract

Although prostate cancer is the second leading cause of cancer death for men in the United States, the genetics of tumor development are poorly understood. Several expressed sequence tagged genes (ESTs) that are expressed predominanlty in the prostate have recently been identified, although their role in the development and maintenance of the prostate is unknown. Here, we demonstrate that the gene identified as UNIGENE cluster Hs. 104215, which codes for a message found predominanlty in the prostate, may be important in tumor development. We name this gene *PCan1* for Prostate Cancer gene 1. Northern blot experiments were performed using RNA isolated from tumor-derived cell lines and human prostate to determine the expression pattern of the gene. DNA sequencing was used to identify mutations that occurred in tumor tissue. By Northern blot analysis, this gene product was not detectable in LNCaP, DU 145, or PC-3 prostate cancer cell lines, although it was readily observed in RNA isolated from total prostate and from dissected central and peripheral regions of prostate. Sequence analysis of genomic DNA from LNCaP, DU 145, or PC-3 cells demonstrated a G/A polymorphism at position 193. Analysis of matched tumor-derived DNA and blood-derived DNA samples from 11 of 13 patients who had undergone a radical prostatectomy and who were homozygous for A in blood-derived DNA demonstrated mutation of position 193 in matched tumor samples resulting in G/A polymorphism. Sixteen additional patient samples were G/A polymorphic in both blood-derived DNA and tumor-derived DNA and two samples were GG in both blood-derived and tumor-derived DNA. Our results suggest that this gene may be a hot spot for mutation in prostate cancer, especially because our radiation hybrid mapping located this gene within a region identified in linkage mapping studies of affected families with prostate cancer. Loss of heterozygosity in prostate tumors has also been reported at the location of *PCan1*. Further studies to determine the functional role of this candidate tumor suppressor gene are warranted.

Key Words: Prostate; tumor suppressor; expressed sequence tag; cell lines; RNA.

Introduction

The prostate is the leading site for cancer incidence, accounting for 31% of new cancer cases in men. It is the second most common cause of cancer death among men 60 yr and older *(1)*.

The genetic events leading to cancer of the prostate are poorly understood. A model of the known molecular changes occurring during prostate cancer progression has been proposed by Pienta and colleagues *(2)*. In this model, normal prostate epithelium progresses to localized prostate cancer, then to

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metastatic prostate cancer, and, finally, to androgen-independent cancer. Proposed early genetic events leading to localized prostate cancer are germline changes, methylation changes, loss of glutathione-*S*-transferase pi gene, loss of heterozygosity of chromosome 8p, and changes in the number of androgen receptor CAG repeats. Proposed changes leading to metastatic disease are loss of chromosome 16q, loss of Rb, loss of KAI, p53 inactivation, and altered E-cadherin expression. Androgen receptor mutations are associated with the progression to androgen-independent cancer. Currently, metastatic disease is essentially incurable.

Germline mutations are estimated to account for approximately 9% of all prostate cancers and 45% of cases in men younger than 55 yr of age. Numerous linkage mapping studies have identified chromosomal regions (1q24–25, 1q42.2–43,1p36, and Xq27–28) that correlate with inherited disease *(3–6)*. A large study of affected brothers with prostate cancer showed suggestive evidence of linkage on chromosomes 2, 12, 15, and 16 *(7)* and candidate regions on chromosomes 5q, 7q, and 19q that provide evidence for linkage to prostate cancer aggressiveness genes. The specific genes involved in prostate cancer development within these large regions are currently unknown. In contrast, the gene HPC2/ELAC2 on chromosome 17p was found to be associated with increased risk for prostate cancer *(8–10)*.

Loss of heterozygosity (LOH) studies of primary cancer tissue have suggested putative tumor suppressor genes located at chromosomes 7, 8, 10, 11, 13q, 16q, and 17. Two known prostate tumor suppressor genes are PTEN/MMAC1, which is predicted to be a protein tyrosine phosphatase *(11)* and TSG101, which is predicted to be a transcription factor *(12)*.

Vasmatzis and colleagues performed a computer-based search of the dbEST database of GenBank, followed by Northern blot analysis to identify three novel expressed sequence tagged (EST) clusters expressed only in prostate *(13)*. Here, we characterize UNIGENE cluster Hs. 104215 for expression in prostate cancer cell lines and for mutation in primary tumor tissue. We demonstrate that this gene is expressed in normal human prostate but is undetectable in three prostate tumor cell lines. We also show that this gene is mutated in tumor-derived DNA obtained from patients at the time of radical prostatectomy. These results suggest that this gene may be important in prostate cancer and for this reason we name this gene *PCan1*.

Materials and Methods

Cell Culture

DU 145, PC-3, and LNCaP human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were cultured as recommended. RNA was isolated using TRIzol (Life Technologies, Rockville, MD) as described by the suppliers.

Animal Prostate RNA Isolation

The use of mice in research was approved by the Institutional Animal Care and Use Committee. Mice were sacrificed by cervical dislocation and the prostate was removed by a trained pathologist with extensive experience in prostate tumor histology. Prostates were immediately homogenized in Trizol (Life Technologies) and RNA was isolated as recommended by Life Technologies. Rabbit prostates were purchased from Pel-Freez Biologicals (Rogers, AR). Rabbits were 1–3 yr old and were fasted for 24 h prior to sacrifice. Tissues were snap-frozen in liquid nitrogen and stored ultracold until use. Tissues were dissolved in Trizol for 5 min and then extracted in chloroform.

Northern Blotting

RNA was electrophoresed through a 1% agarose/2.2 *M* formaldehyde gel and then transferred to a nylon membrane as described by Maniatis et al. *(14)*. IMAGE clone 1011083, which is part of EST cluster Hs. 104215, was purchased from Research Genetics (Huntsville AL), purified by streaking on an Luria Broth (LB) agar plate, and sequenced to verify the correct insert. To generate a ^{32}P-labeled DNA probe, a polymerase chain reaction (PCR) product was generated from purified IMAGE clone 1011083 plasmid DNA using the primers 5′-actcaaggaggctatttatga and 5′-cataaacataagaaacagaggg. The PCR condition was 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by 72°C for 6 min.

The PCR product was purified from a 1% low-melting-point agarose gel. DNA was labeled with ^{32}P by random priming (Life Technologies) and purified

through a PCR purification column (Qiagen, Valencia, CA). Northern blots were prehybridized in ExpressHyb buffer (Clontech, Palo Alto, CA) at 68°C for 30 min. The denatured radiolabeled probe was added to the filter and the filter was hybridized at 68°C for 1 h and then washed for 30 min at room temperature in wash solution 1 containing 2× sodium chloride, sodium citrate buffer (SSC) and 0.05% sodium dodecyl sulfate (SDS). This wash solution was replaced with fresh solution one time. Finally, the filter was washed in buffer containing 0.1× SSC and 0.1% SDS, two times at 55°C for 20 min per wash. Radioactive bands were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant software.

Isolation of Patient Constitutional and Tumor DNA

Patients treated by radical prostatectomy for prostate cancer were enrolled in the study. The study protocol was approved by the Institutional Review Board of the Marshfield Medical Research and Education Foundation and all patients provided informed consent. Constitutional DNA was isolated from whole blood using the procedure of Ciulla et al. *(15)*. Tumor tissue obtained at the time of surgery was fixed in formalin, processed using alcohol and xylene, and embedded in a paraffin block. Sections were cut and analyzed by a pathologist at Marshfield Laboratories and a section of the block containing predominantly tumor was circled, removed, and processed back through xylene and alcohol into saline. Tissues were estimated as >90% tumor by the pathologist using a tissue-dissecting microscope. The tissue was digested with 100 µg/mL proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) in 10 m*M* NaCl, 1 m*M* EDTA, 1% SDS, 10 m*M* Tris-HCl, pH 8.0 at 50°C overnight. Samples were extracted with phenol/chloroform three times and precipitated with 7 *M* ammonium acetate and isopropanol. For sequence analysis, DNA was amplified by PCR and the resulting products were gel purified and sequenced using a ThermoSequenase ^{33}P-labeled terminator cycle sequencing kit (USB, Cleveland, OH).

Radiation Hybrid Mapping

Radiation hybrid mapping was performed by Research Genetics (Huntsville, AL) using the Stanford G3 RH panel. The Stanford G3 RH panel is a medium resolution, 8000-rad panel consisting of 83 clones and two controls. PCR primers were 5′-actcaaggaggctatttatga and 5′-cataaacataagaaacagaggg, which produce an expected PCR product of 200 bp. The same PCR product was used to screen a lambda DASH II genomic library made from human male whole-blood DNA (Stratagene, La Jolla, CA). The library was screened using procedures described by Stratagene.

Results

Vasmatzis and colleagues identified EST cluster Hs. 104215 as a group of ESTs expressed only in the prostate *(13)*. To extend their results and examine the possible role of this gene in prostate cancer, we determined if this gene is expressed in prostate cancer cell lines. In these experiments, total prostate RNA isolated from a male cadaver free of prostate cancer (Clontech, Palo Alto, CA) and total RNA extracted from LNCaP, DU 145, and PC-3 prostate tumor cell lines were examined by Northern blot. Figure 1 shows a dominant mRNA of approximately 600 bp and less intense mRNAs of approximately 1600 and 2400 bases expressed in normal prostate that were not detectable in cell-line-derived RNA. These results are consistent with this gene being involved in the tumorigenesis process. The sizes of the dominant and lesser bands are consistent with the published size of RNA for this gene as determined by Vasmatzis and colleagues *(13)*. Northern blot analysis of oligo-dT-purified RNA from the prostate also resulted in bands of 600, 1600, and 2400 bp, demonstrating that these RNAs are polyadenylated (data not shown). In contrast, Northern blot analysis of total RNA derived from mouse or rabbit prostate did not detect this RNA even though the human positive control and actin controls hybridized as expected (data not shown). We named this gene *PCan1* for Prostate Cancer gene 1.

The prostate is divided into a central and peripheral zone, with the majority of prostate cancers derived from the peripheral region. If *PCan1* is a tumor suppressor gene, it should either be expressed in cells from the normal peripheral zone or function as a gene product delivered to the peripheral zone. Figure 2 shows that *PCan1* is

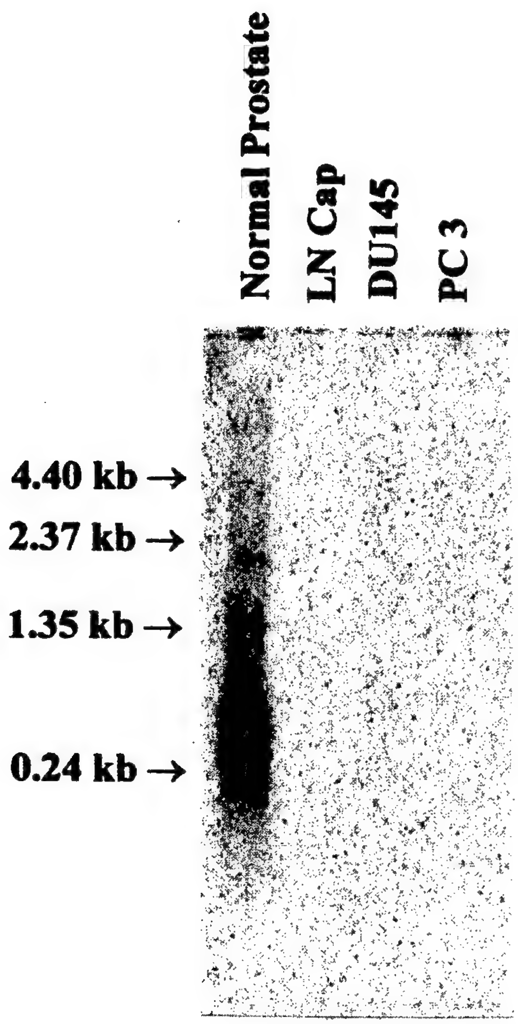


Fig. 1. Northern blot analysis of UNIGENE cluster Hs. 104215. Total RNA (20 µg) isolated from LNCaP, DU 145, and PC-3 prostate cancer cell lines and from a normal adult prostate (Clontech) was electrophoresed through a 1% agarose/2.2 *M* formaldehyde gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA insert excised from plasmid for IMAGE Consortium clone 1011083. Equal loading of RNA samples was demonstrated following hybridization with an actin probe (data not shown). Positions of molecular-weight markers are shown.

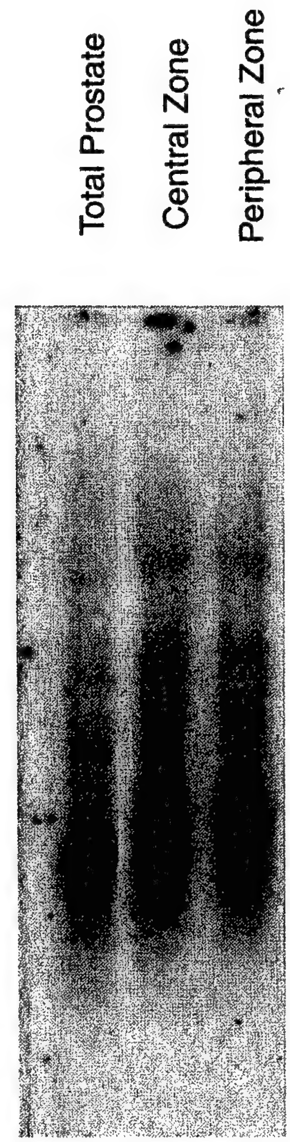
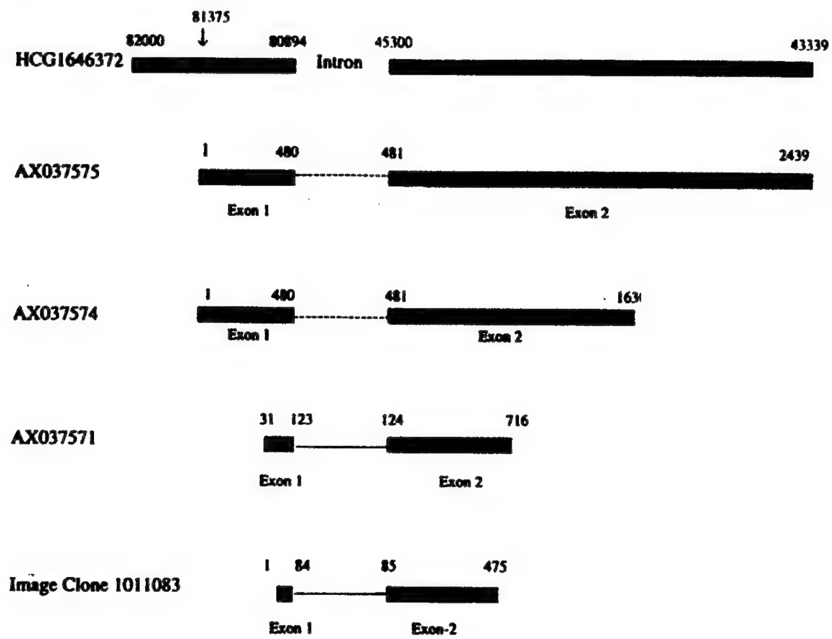


Fig. 2. Expression of *PCan1* in central and peripheral zones of the prostate. Normal human prostate was dissected and RNA was isolated. RNA was electrophoresed through a 1% agarose/2.2 *M* formaldehyde gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA insert excised from plasmid for IMAGE Consortium Clone 1011083. Equal loading of RNA samples was demonstrated following hybridization with an actin probe (data not shown).

expressed in both the central and peripheral regions of the prostate. For these experiments, prostate tissue from a young human male cadaver was dissected into the respective regions and total RNA was isolated. The dissection and RNA isolation were done under contract by Invitrogen (Carlsbad, CA).

To further characterize this gene and predict a possible protein coding region, we sequenced IMAGE

A



B

Image Clone 1011083 sequence

1	ATCTACTACC	GTTTGCTGGT	TTTGAAAATG	GAGAAAAAGA	GTGAGGAACT
51	GAGAAACATG	GATGGCCTTG	GGAACGTGGA	AAAGGGTCAC	TGAAATGGGA
101	CGACATGAAC	TCAAGGAGGC	TATTTATGAC	CATGTCATTT	GCAACATGAA
151	GAAAGCTTAT	CTGGAGTGAA	AGTAAATGAG	ACCAACAGAG	ATAAGAGACC
201	CGGAGAAATC	CTGGTTACAC	TGCTTGAATC	CTGTCAGTCC	TATACTGGAG
251	TCCTGTTAAT	ACAAAATAAT	AGTAATAATC	CCTCTGTTTC	TTATGTTTAT
301	GCCAACTTCA	ACAAAAAGAA	ACTTGACTAA	GAGACAATAT	AAGAACTTAA
351	GTGTAATTAA	GAAAGAACTC	TCCACCACGG	GGAATGTGAA	AGGTATATGA
401	GTCCCTTTTC	ACGATGCGAT	GTCATGTCTT	TTAAATAAGC	CATACTTTAT
451	GTTCAATAAA	AAGAGAATAA	GCAGG		

Fig. 3. **(A)** Gene structure and corrected sequence of *PCan1*. Corrected sequence of UNIGENE cluster Hs. 104215. **(B)** The sequence of IMAGE Consortium clone 1011083 is shown. The underlined base is polymorphic, resulting in either G or A at this position.

clone 1011083. BLAST analysis of GenBank patent database demonstrated that the sequence matched entries AX037571 through AX037575 *(16)*. These entries have sequences of 716 bp (AX037571), 1656 bp (AX037574) and 1632 bp (AX037572), and 2480 bp (AX037575) and 2122 bp (AX037573), respectively. Although details describing the cloning of these cDNAs are not provided in the patent, these clone sizes correspond with the sizes identified on our Northern blots. Consistent with the idea that these sequences code for mRNA, BLAST search of the EST database with the sequence of AX037575 identified IMAGE clones 2733864, 3249202, and 3676688 from the prostate that correspond to the opposite end of the AX037575 sequence than IMAGE clone 1011083. In addition, a BLASTN search of the Celera Publication site *(17)* (website: http://public.celera.com) identified genomic clones hCG 1646372 and clone GA-X2 KMHM RUTHM, which is part of chromosome 4. Figure 3A shows the

predicted gene structure of *PCan1* compiled from the sequences of the genomic clone AX037575, AX037574, and AX03751 and IMAGE clone 1011083. Consistent with this gene structure, a genomic clone isolated from a human genomic DNA library also had an intron with the intron/exon border sequence matching that of the consensus splice site *(18)* (data not shown). Figure 3B shows the corrected sequence of IMAGE clone 1011083.

Radiation hybrid mapping using the Stanford G3 RH panel placed the gene on chromosome 4q near marker D4S2964. D4S2964 is located at 88.35 c*M* on the sex-averaged Marshfield map (http://research.marshfieldclinic.org/genetics). Importantly, this places *PCan1* on chromosome 4q within a 51 c*M* region that is shared between affected sibs who had the mean age of prostate cancer onset in the upper 50th percentile *(7)*. For the marker D4S3243, which is also located at 88.35 c*M*, a *P*-value of $0.05 > p > 0.01$ was obtained between the respective Z_{1r} scores of the subgroups. In addition, loss of heterozygosity has been reported for 4q in prostate adenocarcinomas *(19)*. These results suggest that *PCan1* is a susceptibility gene for prostate cancer that exerts its effect later in life.

To investigate the possibility that *PCan1* is mutated in prostate tumor tissue, we amplified and then sequenced genomic DNA from matched tumor and blood samples obtained from patients at the time of radical prostatectomy. For these studies, tumor tissue was fixed, sectioned, and examined by a pathologist to determine the normal versus neoplastic margins. Only samples estimated at greater than 90% tumor were used for these studies. Sixteen percent of the patients were younger than 60 yr at the time of surgery. Sixty-two percent of the cases were stage II, 35% were stage III, and 3% were stage IV.

Figure 4 shows the DNA sequence from an individual's blood-derived DNA and tumor-derived DNA. The mutated nucleotide is highlighted. Table 1 shows that 11 of 13 patients whose inherited genotype was homozygous A had mutations in tumor DNA, resulting in an A/G polymorphism. Sixteen patients inherited the A/G polymorphism genotype. Additional sequencing using DNA derived from LNCaP and DU 145 prostate cancer cell lines demonstrated that position 193 of the DNA sequence was heterozygous in both cancer cell lines, resulting in either an A or G.

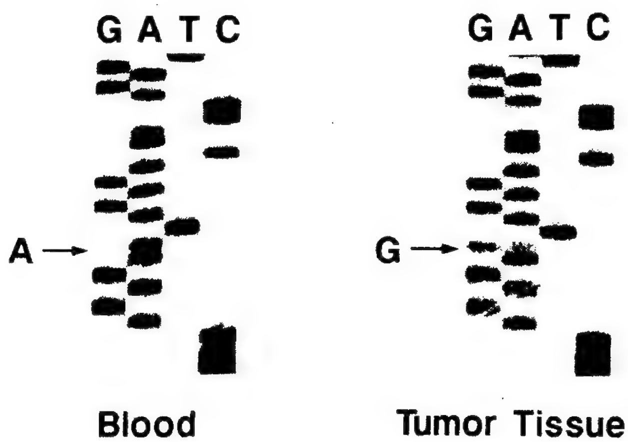


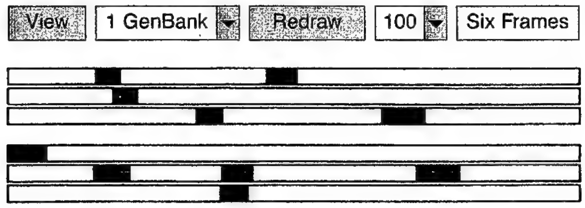
Fig. 4. Analysis of *PCan1* in tumor and blood genomic DNA. Matched tumor and blood genomic DNA samples were amplified by PCR and sequenced. The arrow marks the base that is mutated in tumor tissue.

Table 1
Summary of Patient and Tumor Polymorphisms

Blood→Tumor	Number	Genotype
AA→AA	2	Nonmutated
AA→AG	11	Mutated
AG→AG	16	Heterozygous
GG→GG	2	Homozygous

Note: Genomic DNA isolated from tumor and matched blood samples was analyzed for inherited polymorphism status and mutations. The altered amino acid is highlighted in Fig. 3B.

To identify possible protein coding regions within *PCan1*, we analyzed the sequence of AX037575 using an open reading frame finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), which is an analysis tool that finds all open reading frames in a user's sequence. We used the sequence of AX037575, as this is the largest sequence and it encompassed the sequences of the other clones. Figure 5 shows the predicted open reading frames. Significantly, the largest open reading frame is 192 nucleotides, which would code for a protein of 63 amino acids. However, this protein is not synthesized from the first AUG translation codon and the translation start site does not coincide with the consensus sequence *(20)*, suggesting that this may not be an actual protein prod-



Frame	from to	Length
+3	1620..1811	192
-2	1775..1963	189
-1	3.. 170	168
-2	377.. 529	153
+1	1120..1254	135
-2	926..1060	135
-3	919..1041	123
+3	816.. 929	114
+2	458.. 565	108
+1	385.. 489	105

Fig. 5. Analysis of sequence AX037575 for protein coding regions. To identify possible protein coding regions within *PCan1,* we analyzed the sequence of AX037575 using open reading frame finder. The possible open reading frames are shown with the nucleotide positions and length.

uct. A combination of gene expression, in vitro translation, and immunoprecipitation experiments will be necessary to confirm or refute this protein product. Neither BLAST analysis of the nonredundant sequences in GENBANK nor a search of the PROSITE database showed proteins or RNAs of significant homology, suggesting that this gene may have a unique biological function. Vinals and Klee *(16)* identified the same putative open reading frame of 63 amino acids, but they did not provide experimental data in support of this sequence.

Discussion

We have characterized a novel gene, *PCan1,* which may function as a prostate cancer tumor suppressor gene. *PCan1* is predominantly expressed in prostate tissue and is undetectable in the prostate cancer cell lines we tested. *PCan1* is located at 4q21, a chromosomal region previously shown to be shared by affected sib pairs with late-onset prostate cancer *(3,7).* Significantly, *PCan1* is mutated in approximately 35% of prostate adenocarcinomas.

The role of *PCan1* in prostate cancer development is an important question. Vinals and colleagues *(16)* used real-time PCR to analyze the expression of *PCan1* in 3 normal prostate samples, 22 prostate tumors, 2 metastatic tumors, and 3 benign prostatic hyperplasia samples. Although they did not publish the data in their patent, they suggest that *PCan1* was highly expressed in approx 93% of normal prostate and prostate tumor samples tested and retina. They did not provide results or analyses regarding the Gleason grade of the samples or patient demographics such as age, race, or tumor metastasis. Furthermore, Vinals and colleagues *(16)* did not report any results regarding possible mutation of *PCan1* in human tumor tissue or the correlation of mutations with expression level.

Little information regarding the function of *PCan1* is available through database searches for related genes. Sequence analysis of *PCan1* did not identify an obvious open reading frame. Several examples of expressed mRNAs lacking an open reading frame have been described *(21).* Two prostate-specific examples include PCGEM1 *(22)* and DD3 *(23).* PCGEM1 and DD3 are prostate-specific genes that are overexpressed in prostate cancer and may function as part of an emerging class of noncoding RNAs termed "riboregulators." Each of these mRNAs function as regulatory molecules affecting the availability of their complementary sequence. It is currently unknown what biological effect the A to G mutation causes. This mutation may affect RNA folding and stability. It is also unclear at this time if the gene functions early in the initiation of tumorigenesis or at a later stage of cancer, such as progression or metastasis.

Several studies have identified novel genes expressed solely or predominantly in prostate tissue based on differential RNA screening techniques or computer searches of the available gene databases *(13,23–27).* The tissue-specific expression pattern suggests that these genes may either function as prostate-specific tumor suppressor genes or that they may have a role in the establishment of the prostate as an organ and may specify prostate-specific cell types or activities.

PCan1 is not expressed in normal prostate tissue from mouse or rabbit (data not shown). Similarly,

prostate-specific membrane antigen is primarily expressed in normal human prostate, but was not expressed in mouse prostate, although it is detected in mouse brain and kidney tissue *(28)*. In contrast, α_2-microglobulin is expressed in the male rat, but is not detectable in humans *(29)*. Consistent with these paradoxical results, the histologies of human and animal prostates are different, with human prostate basal cells forming a continuous layer, whereas, in animals, basal cells form a sporadic layer of pseudostratified epithelium *(30)*. Determining the role of *PCan1* in the growth, differentiation, and function of human prostate is essential.

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Genetics of Prostate Cancer

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Running Head: Prostate genetics

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ABSTRACT

Prostate cancer is the most frequently diagnosed visceral cancer of men, responsible for approximately 40,000 deaths in adult males per year. To identify the genetic causes of prostate cancer, we performed a whole genome scan of affected sib pairs, using DNA markers spaced evenly across the human genome. We demonstrated that regions on chromosomes 1, 4, 5, 7, 8, 11, 16 and 19 might harbor genes that predispose individuals to prostate cancer and may affect tumor growth rate and tumor aggressiveness. Here we present DNA sequence analysis of KIAA 08782 and 17β-hydroxysteroid dehydrogenase that are located on chromosome 16 within the mapped region, and we demonstrate that neither of these genes carries mutations in the protein coding region or their splice junction sites. These results suggest that these genes are less likely to be associated with the cause of familial prostate cancer.

INTRODUCTION

Prostate cancer is the second leading cause of cancer mortality for men in the U.S. with a rate of 26 deaths per 100,000.[1] Although prostate cancer is potentially curable in its early stages, treatment of advanced refractory hormone prostate cancer is ineffective, making early detection and treatment of disease a necessity.[2] Unfortunately, approximately 30% of men with prostate cancer have tumor that has spread beyond the prostate gland at the time of diagnosis. Despite the impressive response of metastases to androgen hormone deprivation, the survival rate is dismal, with greater than 90% of these patients dying from their cancer[3]. Screening tests for prostate cancer include digital rectal examination, transrectal ultrasonography, and measurement of prostate-specific antigen. However, these tests often do not detect the tumors before they have spread. The development of additional tests for earlier identification of prostate tumors will be extremely important.[4,5]

One of every six men will be diagnosed with prostate cancer in his lifetime. Factors that increase a man's risk for prostate cancer include increasing age, race, family history and lifestyle. Men over the age of 65 are at the highest risk, yet 25 percent of cases are diagnosed under the age of 65. African-Americans have the highest rate and are 50% more likely as white males to be diagnosed with prostate cancer and twice as likely to die from this disease.[6]

Continuous androgen stimulation is necessary for development and maintenance of the prostate gland. Thus, genes that affect the synthesis and metabolism of androgens may modulate risk for prostate cancer or the tendency of a tumor to progress to the more aggressive forms of this disease.[7] Similarly, genes regulated by androgens are also likely to be important in prostate cancer development.

17β-hydroxysteroid dehydrogenase (HSD) is expressed in prostate and also extragonadal tissues, including liver and kidney and catalyzes the conversion between the low activity sex steroids, androsteinedione and 5α-androstanedione, and the more potent forms, testosterone and 5α-dihydrotestosterone.[8] 17β-HSD activity is essential for testosterone synthesis in the gonads and may also regulate the concentration of active sex steroids locally in target cells such as prostate. 17β-HSD is most efficient in the oxidative inactivation of testosterone and 5α-androstanedione to their less active 17-keto metabolites. Our hypothesis is that mutations or polymorphisms that inactivate or significantly reduce the activity of 17β-HSD would increase androgen levels leading to increased hyperplasia within the prostate and ultimately more cancer due to the increased proliferation of cells.

A model of the known somatic mutations occurring during prostate cancer progression was proposed by Pienta and colleagues.[9] In this model, normal prostate epithelium progresses to localized prostate cancer, then to metastatic prostate cancer, and finally to androgen-independent cancer. Proposed early genetic events leading to localized prostate cancer are germline changes, methylation changes, loss of glutathione-s-transferase pi gene, loss of heterozygosity (LOH) of chromosome 8p and changes in the number of androgen receptor CAG repeats. Proposed changes leading to metastatic disease are loss of chromosome 16q, loss of Rb, loss of KAI, p53 inactivation, and altered E-cadherin expression. Androgen receptor mutations are associated with the progression to androgen independent cancer. Mutations of PTEN, a protein tyrosine phosphatase[10], and of TSG101, a putative transcription factor[11], are frequent in prostate tumors. LOH studies of primary tissue have identified putative tumor suppressor genes located at chromosomes 4, 7, 8, 10, 11, 13q, 16q, and 17.[12-22]

The genetics of inherited prostate cancer are poorly understood. Family history of prostate cancer is a strong predictor of disease with inherited germ line mutations being estimated to account for approximately 9% of all cancers and 45% of cases in men younger than 55.[23,24] Linkage mapping studies have identified chromosomal regions 1q24-25, 1q42.2-43, 1p36, Xq11, Xq27-28, 20q13 and 11p that correlate with inherited disease.[25-31] The specific genes involved in prostate cancer development within these regions are largely unknown. Mutation of RNASEL at 1q24-25 segregate with two HPC-1 linked families.[32] However, mutations were not identified in other families linked to HPC1 and inactive RNASEL alleles are present at low frequency in the general population. The gene HPC2/ELAC2 on chromosome 17p was found to be associated with increased risk for prostate cancer.[33,34] However, analysis of this gene in families with prostate cancer failed to show excess clustering of the abnormal allele, suggesting that the amino acid change does not cause prostate cancer.[35]

To identify genes that predispose families to prostate cancer, we have performed a linkage mapping study of affected sib pairs.[36] In this study, we collected blood-derived DNA from approximately 600 affected sib pairs identified at Washington University in St. Louis. For each patient, we recorded the vital statistics such as age, family history of prostate cancer and breast cancer, and tumor stage and grade at diagnosis. DNA samples were tested at the Marshfield Medical Research and Education Foundation using DNA markers spaced evenly across the human genome. Analysis identified the highest probability score on the long arm of chromosome 16 when the data were analyzed without stratification. Regions identified following stratification of the patient population according to various clinical parameters include 1p35.1 for families with breast cancer, 4q in late-age-at-onset patients, and 5q, 7q, and 19q in patients with aggressive disease.[37]

Here we report sequence analysis data regarding two genes within the region of interest on chromosome 16. For these studies, DNA samples were sequenced from patients likely to carry a mutated gene in this region and from control patients who did not show significant association with this chromosomal region. Our results do not find a causative mutation in either of the candidate genes 17β-hydroxysteroid-dehydrogenase (17β-HSD) or KIAA 0872, a cDNA clone of unknown function, and they demonstrate that additional DNA sequencing within the mapped region on chromosome 16 is necessary to find the DNA sequence that predisposes patients to prostate cancer.

MATERIALS AND METHODS

Families were ascertained from Washington University Medical School when two or more documented cases of prostate cancer were present in the family. The Institutional Review Boards at both Washington University Medical School and Marshfield Medical Research Foundation approved the research protocols and all patients provided informed consent. The diagnosis of prostate cancer was confirmed directly by a pathologist or through examination of medical records. Determination of age-at-diagnosis and health status of other family members was made from examination of medical records. Tumors were graded using the Gleason System. All patients were Caucasian.

The coding region of KIAA 0872 was sequenced to determine if mutations were present that might contribute to the development of prostate cancer. Genomic DNA extracted from blood samples of 18 prostate cancer patients with high frequency of allele sharing on 16q and blood samples from 18 prostate cancer patients with a low-frequency of allele sharing on 16q were sequenced along with 3 prostate cancer cell lines (DU145, LNCaP, PC-3) and 9 genomic DNAs from donors without prostate cancer. Patients with a high-frequency of allele sharing are

those in which a sib pair is likely to share both of their extended haplotypes identical-by-descent, whereas low-frequency means that a sib pair is inferred to share neither haplotypes identical-by-descent. Five exons of KIAA 0872 were determined by aligning the cDNA sequence (GenBank accession # AB020679) with the chromosome 16 genomic sequence (GenBank accession # AC009139) using the BLAST 2 Sequences program from National Center for Biotechnology Information. (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The exons were amplified by polymerase chain reaction using the oligo primers shown in Table 1. Exons 1, 2, 4, and 5 were amplified entirely in one reaction with one primer set. Exon 3 was amplified in segments with multiple primer sets (see Table 1). Amplification products of the expected size were excised and purified from a 1% agarose gel. The purified amplification products were sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, OH) with the primers shown below. Sequences were analyzed for mutations by electrophoresis through a 6.5% polyacrylamide sequencing gel.

The exons, as described by Labrie and colleagues[38], of the human type II 17β-hydroxysteroid dehydrogenase gene (17β-HSD) were analyzed for mutations potentially contributing to prostate cancer. Exons from the genomic DNA of one normal donor, 3 prostate cancer cell lines (DU145, LNCap, PC-3) and 22 prostate cancer patients with low-frequency of allele sharing on 16q and 22 prostate cancer patients with high-frequency of allele sharing on 16q were amplified and sequenced (as described previously) with the primer sets shown in Table 2.

Additionally, the 5' untranslated region presumably containing the 17β-HSD promoter was screened for mutations predisposing to prostate cancer. Segments of the promoter region were amplified and sequenced as described previously with primer sets listed in Table 3.

Differences in the expression levels of both the KIAA 0872 gene and 17β-hydroxysteroid dehydrogenase gene between normal and cancerous prostate cells were examined by Northern hybridization of total RNA from normal prostate cells, 3 normal prostate cell lines and 4 cancerous prostate cell lines. Total RNA from normal prostate was purchased from Clontech (Palo Alto, CA). Normal prostate cell lines (PZ-HPV7 and PNT2) and cancerous prostate cell lines (DU145, LNCaP, PC-3 and Ca-HPV10) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Total RNA was extracted from cells using Trizol reagent as described by the manufacturer (Life Technologies, Inc., Rockville, MD). Four micrograms of total RNA for each sample mentioned above was equalized for β-actin signals prior to loading on formaldehyde electrophoresis gels (as described in Molecular Cloning, 3rd Ed., by Sambrook and Russell). Total RNA, after electrophoretic separation, was transferred and UV-crosslinked to a nylon membrane. Hybridization probes were produced by amplifying Exon 5 of KIAA 0872 and by excising the insert from Image Clone for 17β-HSD. Probes were labeled with α-^{32}P-dCTP (NEN, Boston, MA) using the Random Prime Labeling Kit (Life Technologies, Inc., Rockville, MD). Nylon membrane carrying total RNA was prehybridized in ExpressHyb Hybridization Buffer (Clontech) for 30 minutes at 65°C. Buffer was replaced with fresh ExpressHyb buffer containing 1×10^6 cpm of heat denatured α-^{32}P labeled probe and hybridization carried out for 60 minutes at 65°C. The membrane was rinsed with wash buffer containing 2x sodium chloride, sodium citrate buffer (SSC) and 0.05% sodium dodecyl sulfate (SDS) and then washed 2 x 15 minutes at room temperature with continuous shaking. Two additional washes at 56°C were performed in wash buffer containing 0.1X SSC, 0.1% SDS. The membrane was rinsed briefly with 2X SSC and exposed to phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Northern hybridization with a human type II 17β-hydroxysteroid-dehydrogenase probe showed significant decreases in the amount of 17β-HSD message in the cancer cell lines, LNCaP, DU145, PC-3 as compared to the normal prostate message (Figure 1). The cancer cell line, Ca-HPV10, showed a slightly increased level of expression versus the normal prostate. One of the two normal prostate cell lines, PNT2, showed expression levels similar to the normal human prostate message but the message from the normal human prostate epithelial cell RNA (Clonetics) was significantly decreased. Based on these results, we sequenced genomic DNA from prostate cancer patients showing genetic linkage to chromosome 16q to identify inherited mutations or polymorphisms that may cause reduced 17β-HSD expression.

Mutation analysis of 17β-hydroxysteroid-dehydrogenase revealed one single nucleotide polymorphism in each of exons 2, 5, 6, 7 and in the 5' untranslated region of exon 1. Three patient samples with high-frequency allele sharing on chromosome 16q had the a -> g polymorphism in exon 2. All the other samples corresponded to the published sequence being homozygous for g. The a -> g single nucleotide polymorphism in exon 2 did not change the amino acid at this position. One control sample showed a t -> c polymorphism in exon 5, but none of the cancer patient samples had this polymorphism. Three samples had an a -> g transition in exon 6: the LNCap cell line and two patients with low frequency of allele sharing. This polymorphism produced a change in the resulting amino acid from methionine to valine. However, when an additional 12 high frequency and 12 low frequency of allele sharing samples were sequenced in this region, no additional samples carried this polymorphism. Therefore, only one cell line and 2 of 44 (4.5%) prostate cancer patient samples carry this polymorphism. One

high frequency of allele sharing patient sample had a null mutation in exon 7. This g -> a polymorphism did not produce a change in the amino acid.

A c -> t polymorphism in the 5' UTR of exon 1 was identified in 40% of samples tested (10 of 20 high-frequency and 6 of 20 low-frequency samples). Mutations in the 5' UTR have the potential to alter protein translation rates. Of the samples, 17.5% (2 of 20 high-frequency and 5 of 20 low-frequency samples) were homozygous t at this position and 42.5% of samples (8 of 20 high-frequency and 9 of 20 low-frequency samples) showed no polymorphism or mutation, remaining homozygous c at this position.

KIAA 0872 is a novel gene located within the region of linkage on chromosome 16q. Blast analysis of the protein sequence of KIAA 0872 against the sequences in the Swiss Pro protein database failed to show homology between KIAA 0872 and any known proteins (data not shown). Northern results demonstrated expression of KIAA 0872 in normal prostate and each of the cell lines (Figure 2), suggesting that sequence analysis of KIAA 0872 for mutations in prostate cancer are warranted. The two bands identified in the Northern blot (Figure 2) are consistent with the two cDNAs catalogued in GenBank and likely represent alternate splicing of the same gene.

Mutation analysis of KIAA 0872 revealed two polymorphisms in exon 3. Exons of the KIAA 0872 gene were sequenced in prostate cancer genomic DNA from 18 patients with a high-frequency of allele sharing on chromosome 16 and 18 patients with a low-frequency of allele sharing in an effort to locate mutations in the protein coding region. In addition, 9 control DNA samples, whose prostate cancer status is unknown to the investigators and 3 prostate cancer cell lines were sequenced. The two sites of polymorphism, one at base 1144 and the other at base 1249, were noted with a single base substitution on one or both strands of genomic DNA. None

of the base substitutions found resulted in a change in the amino acid coding sequence (summary of polymorphisms is in Table 4). Interestingly, the polymorphism at base 1249 of the mRNA sequence seems to segregate with families; families 4013 and 1349 of the high-frequency samples show this polymorphism as does the low-frequency sample of family 1349. Family 1349 is a trio of affected sibs, two of whom are identical over the region of interest while the third appears to share neither of his haplotypes with his other two sibs. Two control samples carried the TC polymorphism at base 1249 as did both samples of family 1751 in the low-frequency samples. In total, 2 prostate cancer families in both high- and low-frequency categories of allele sharing in 16q showed segregation of the TC polymorphism at base 1249 of KIAA 0872 (3' end of exon 3).

DISCUSSION

Because prostate cancer is a very common cause of disability and death in men, studies of the genetics of this disease are very important. A recent analysis of family and twin studies for cancer concluded that genes contribute high risks for most cancers and that early age at diagnosis is associated with increased familial disease.[39] Consistent with this, our previous linkage mapping study of affected sib pairs identified at least 8 different chromosomal regions that may be important in prostate cancer development.

Of the methods currently available for the identification of genes that cause disease, we chose the candidate gene approach. This approach utilizes available information on the function of genes to choose those that may be most likely to carry familial mutations. Because of the pivotal role of androgens in the formation and maintenance of the prostate gland, we first analyzed 17β-HSD. We also chose to study KIAA 0872 since the amino acid sequence predicts a novel protein that is unique from other known protein families.

Our results did not identify mutations in the coding region of either of these genes, and the polymorphisms that were identified did not occur in affected individuals at a higher frequency than the controls. We also did not identify mutations in the intron/exon splice sites. However, we cannot rule out the possibility of mutations in the introns or the promoter region of these genes. Most cancer genes identified to date have mutations within the protein-coding region in some of the of affected individuals.

Several laboratories have reported loss of heterozygosity (LOH) on 16q[19,40,41] near the region identified by linkage mapping of affected families. LOH is the loss of a region on one copy of the chromosome and frequently occurs at the site of a tumor suppressor gene. As a result of LOH, a patient with one inherited allele becomes null for the tumor suppressor gene due to deletion of the second allele. In a recent study, the putative location of the tumor suppressor gene on chromosome 16q has been narrowed using LOH analysis of tumors[42] suggesting that it may be possible to refine the search for the prostate cancer gene using this information. Consistent with the LOH results, the fragile site on chromosome 16 has the same location.[43] Whether the increase in LOH at this location on chromosome 16 is the result of chromosomal breaks at the fragile site or the actual presence of a tumor suppressor gene that is lost during tumor growth needs to be determined. Neither 17-β-HSD nor KIAA 0872 are within the narrow region identified by LOH, although they are near the peak identified by linkage mapping.

Our replication study of chromosome 16q using an additional group of prostate cancer patients collected at the Marshfield Clinic, University Hospitals of Cleveland and Cleveland Clinic and Washington University Medical School did not add evidence of linkage in this region to a prostate cancer gene.[44] However, independent analysis of families collected at the Marshfield Clinic did support 16q as the location of a gene in familial prostate cancer

(unpublished data). In contrast, other whole genome scans of affected patients did not provide evidence of a gene on 16q.[25,29] The differences in these results are likely to be due to heterogeneity in the genetic background of the patient population, assessment criteria for enrollment of families, and methods used for statistical analysis.

Compelling studies suggest that multiple genes are involved in the development of prostate cancer. Although it has proven difficult to date to identify the exact mutations, further studies are indicated to explore the possibility for early diagnosis and treatment of prostate cancer based upon the genetic constitution of the patient.

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Table 1: PCR and sequencing oligo primers for mutation analysis of KIAA 0872.

Exon	Primer Set	Forward Primer	Reverse Primer
1		5'- ctc gat tccg cct ccc act	5'- gga ttc ctt tta aga ccc cac t
2		5'- aca tgc agt gac cca cca g	5'- cag tca tgt ccc cca ttc t
3	A	5'- ccc cct tct tac ccc ttc tt	5'- gtc cag aag tcg gtc cag
	B	5'- act atg acc tcc gcc gcc	5'- ccg cct act gcc agc act
	C	5'- gcg ctc ctc cga cgt tg	5'- agg cag aca ttc ata gca
	D	5'- aac cct gat ggt ttt ttc t	5'- ggc gcc cga cag cct gca
	E	5'- cct cat cag cca gtg ctc	5'- acc cag cca gtg cct tag gg
4		5'- cct gga cta acc ttg tcc	5'- tca ctc aga gcc ctg ccc
5		5'- cca gct ctg gcc atc tga	5'- ccc aca cac att tcc ggg

Table 2: PCR and sequencing oligo primers for mutation analysis of 17β-Hydroxysteroid Dehydrogenase.

Exon	Forward Primer	Reverse Primer
1	5'- cct tgg tat tta tgt tga a	5'- tca gtg aaa att gta gca
2	5'- cct gtc act ctg gtt tga	5'- gag tgg ggg ggc atg ttc
3	5'- att cac ttc ctc ttc ca	5'- atg cca tga gcc atg tct t
4	5'- ttc ctc tct tat aag aat g	5'- gtt gag tga att gct ccc
5	5'- aat aag tcc ttt ctc aca	5'- cag gcc ctg atc ttc tag
6	5'- ttt att gga tga aca aat	5'- cag acc aag agg acc ttc
7	5'- ctt ccc aac aga gac aag	5'- aaa gta cta ttc aat caa

Table 3: Amplification and sequencing oligo primers for mutation analysis of putative promoter region of 17β-Hydroxysteroid Dehydrogenase.

Primer Set	Forward Primer	Reverse Primer
1	5'- gat tag att tga ata gag gg	5'- agt ctt gca gcc tga tct ca
2	5'- gaa tag aat gct tag ggg ca	5'- agg gat atg aaa gta gag ga
3	5'- gac caa agc act ttc ctc ta	5'- atg aca gtc cac ctc tgg ag
4	5'- ccc tgg cat gtt tcc act cag gc	5'- gcc tga gtg gaa cat gcc a

Table 4: Summary of Polymorphisms found in Exon 3 of KIAA 0872 mRNA sequence. Bases shown in bold are those segregating with families.

Sample Frequency	Sample	base 1144	Amino Acid	base 1249	Amino Acid
Standard	GenBank sequence	CC	Valine	TT	Valine
Control	1			CC	Valine
Control	2			CC	Valine
Control	3	CC	Valine	CC	Valine
Control	4			CC	Valine
Control	5			**TC**	Valine
Control	6			CC	Valine
Control	7			**TC**	Valine
Control	8			CC	Valine
Control	9			CC	Valine
Cell line	DU 145	CC	Valine	N/A	
Cell line	LN CaP	N/A		CC	Valine
Cell line	PC-3	CC	Valine	CC	Valine
High	4258-03	TC	Valine	CC	Valine
High	4258-05	TT	Valine	N/A	
High	4013-03	CC	Valine	**TC**	Valine
High	4013-04	CC	Valine	**TC**	Valine
High	1886-18	CC	Valine	CC	Valine

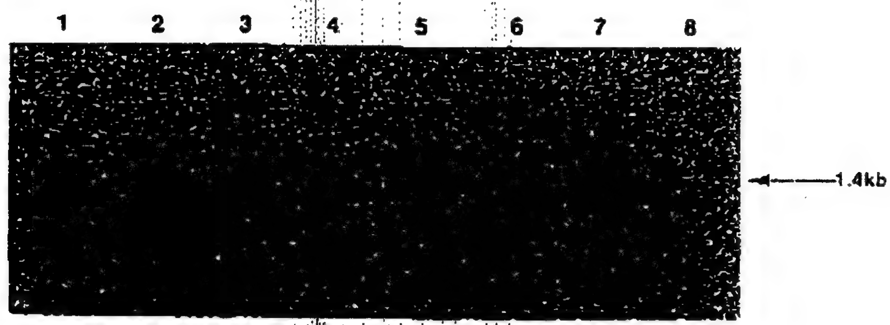
High	1886-19	CC	Valine	CC	Valine
High	1349-03	CC	Valine	TT	Valine
High	1349-04	CC	Valine	TT	Valine
High	4250-03	TT	Valine	CC	Valine
High	450-04	TT	Valine	CC	Valine
High	4171-03			CC	Valine
High	4171-04			CC	Valine
High	3167-03			CC	Valinc
High	3167-04			CC	Valine
High	3212-04			CC	Valine
High	3212-05			CC	Valine
High	4041-03			CC	Valine
High	4041-04			CC	Valine
Low	4413-05	TC	Valine	CC	Valine
Low	4413-03	TT	Valine	CC	Valine
Low	4143-05	TT	Valine	CC	Valine
Low	4143-03	CC	Valine	CC	Valine
Low	3790-05	TT	Valine	CC	Valine
Low	3790-03	CC	Valine	CC	Valine
Low	4010-04	TC	Valine	CC	Valine
Low	1349-03	CC	Valine	TT	Valine
Low	1349-05	TC	Valine	TC	Valine
Low	1349-04	CC	Valine	TT	Valine

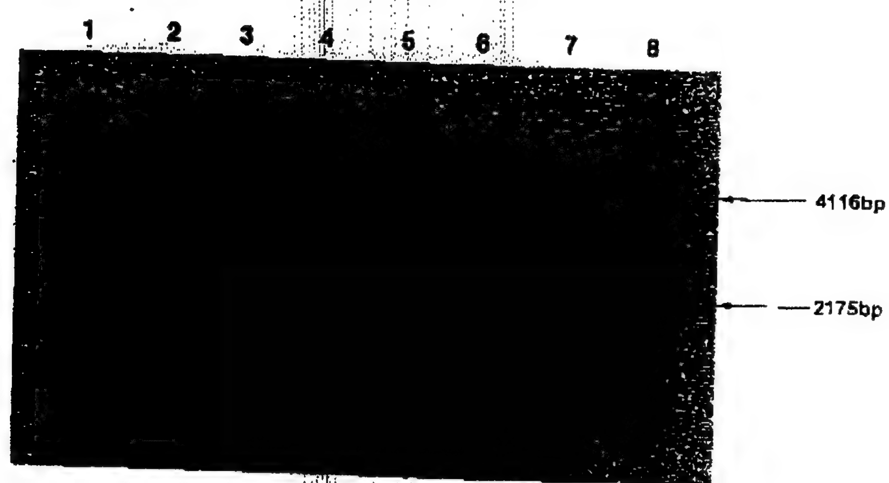
Low	3766-04			CC	Valine
Low	3766-03			CC	Valine
Low	1911-04			CC	Valine
Low	1911-03			CC	Valine
Low	1751-03			TC	Valine
Low	1751-04			TC	Valine
Low	4209-04			CC	Valine
Low	4209-03			CC	Valine

LEGEND TO THE FIGURES

Figure 1. Northern analysis of 17β-HSD. Samples are 1) Total human prostate, 2) LNCAP, 3) Du145, 4) PC-3, 5) PZ-HPV-7, 6) Ca-HPV-10, 7). PNT2, 8) Clonetics normal epithelial cells.

Figure 2: Northern analysis of KIAA 0872 expression in prostate. Samples are the same as Figure 1.


1 2 3 4 5 6 7 8
1.4kb


1 2 3 4 5 6 7 8
4116bp
2175bp

Methylenetetrahydrofolate reductase variants and risk of familial prostate cancer.

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Background. Methylenetetrahydrofolate reductase (MTHFR) variants, with decreased enzymatic function, are implicated in the increased risk for neural tube defects and a slight decrease in risk for colon cancer and acute lymphocytic leukemia. MTHFR is important in both DNA synthesis and methylation. *MTHFR* maps to 1p36, a region of reported linkage to familial CaP in families in which other tumor types, such as breast and brain cancers, segregate. A recent study by Kimura and colleagues suggested a trend between the exon 4 variant and poorly differentiated tumors.

Methods. We genotyped 2 MTHFR variants at amino acid position 222 (exon 4) and 429 (exon 7) in 613 men affected with prostate cancer (CaP) from 292 families and in 562 controls. The data were analyzed for association as well as for linkage.

Results. Hardy-Weinberg equilibrium existed in the controls. There was no association between CaP and either the exon 4 or the exon 7 polymorphism. Additionally, neither Gleason score nor tumor stage demonstrated an associationwith *MTHFR* variants. Allele sharing was not seen in single-point linkage analysis for either mutation. Considerable linkage disequilibrium was observed. Surprisingly, 7 chromosomes in controls and 1 chromosome in cases had the variant allele at both sites.

Conclusion. Our results do not support involvement of MTHFR variants in the etiology of CaP. Unlike Kimura et al., (2000), we did not observe a trend between the variant allele at exon 4 and

Keywords: methylenetetrahydrofolate reductase, familial prostate cancer, association study, genetic analysis.

Introduction

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in both DNA synthesis and methylation; therefore, its activity affects DNA stability and gene expression. MTHFR is a flavoprotein dimer responsible for generating the circulating form of folate, 5-methyltetrahydrofolate (5-methylTHF) by reduction from 5,10-methylenetetrahydrofolate (5,10-methyleneTHF). The 5,10-methyleneTHF, is a substrate for purine and thymidine synthesis, while 5-methylTHF is necessary for methionine synthesis, which, in turn, is a substrate for S-adenosyl methionine (SAM). SAM is the universal methyl donor in methyl transfer reactions including DNA methylation. High MTHFR function results in low 5,10-methyleneTHF, causing dUTP incorporation into DNA, thereby producing double stranded breaks (1).

The gene for *MTHFR* is located on chromosome 1p36. It has 11 exons (2) and a number of single nucleotide polymorphisms (SNPs) within the coding region. Four SNPs change the amino acid sequence of the protein, while another 4 are synonymous substitutions (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=4524). Two of the SNPs affect MTHFR's function. One, a C to T change at nucleotide 677 (C677T), changes an alanine at position 222 to valine and affects the catalytic domain of the enzyme (3). This form of the protein is thermolabile and has a reduced activity. The enzyme levels are only 30% of the "wild type", and individuals homozygous and heterozygous for this mutation have an increased amount of homocysteine (4). Folate acts to stabilize the protein and neutralize the effects of the mutation (4). The folate can be ingested, either as a supplement or by consumption of fruits and vegetables. The frequency of the variant is heterogeneous in different regions of the world; the lowest frequency is found in sub-Saharan Africa and northern Canada and the highest in Southern Europe and South America (5, 6). The second variation is an A to C transversion at 1298 (A1298C), which changes a glutamate to alanine at position 429. This amino acid is within the regulatory domain of the protein (7). By

itself it does not seem to affect the enzyme's function, but in trans with the C677T allele, the enzymatic levels of MTHFR are reduced (8, 9).

A number of studies have reported an effect of the *MTHFR* polymorphisms and the modification of tumor development risk. Skibola et al. reported a decreased frequency of either of the 2 homozygous variant alleles (i.e. 677TT or 1298CC) in patients with acute lymphocytic leukemia, though not in acute myeloid leukemia (10). A similar decrease was reported by a number of investigators among patients with colorectal cancer (reviewed by (11). However, Houlston and Tomlinson included a caveat that most of the studies were not large enough to detect effects 2-fold in size, while one report (12) may have reported an effect of the MTHFR variants that was a result of population stratification. The meta analysis of the combined data though, could not reject the trend of decreased 677TT frequency in the patients with colorectal tumors.

In contrast, Song et al., reported a 6-fold increased risk of developing esophageal squamous cell carcinoma (ESCC) in patients that were homozygous for the 677T allele compared to those homozygous for the 677C allele in a large Chinese case-control study. Furthermore, they reported an elevated risk for ESCC development associated with the 677 mutation in an allele-dose relationship (13). Finally, they found an indication of increased risk with the 1298C mutation, though this effect was smaller (the authors suggest that it was due to the lower allele frequency of 1298C in the Chinese population). In a case-control study of Chinese gastric cancers, Shen et al., found a minimal increased risk of tumor development in patients who were 677TT, but not in those who were 1298CC (14).

Prostate carcinoma has been reported to have alternations in DNA methylation (reviewed in (15) including hypermethylation (16, 17) and hypomethylation in many advanced tumors (18). Imbalance in folate and methyl group metabolism brought on by dietary deficiency, or genetic predisposition, has been suggested to have an effect in prostatic carcinoma (19). Kimura et al.,

examined the 677 polymorphism in a sample of 132 German patients and 150 controls (20). They did not find significant correlation between the *MTHFR* genotype and CaP, though the patient population showed a slight increase of the variant form of the protein. Examination of genotype distribution with clinical parameters also did not yield significant findings, but again, the *MTHFR* 222val allele was more prevalent in patients with poorly differentiated tumors (P = 0.052 in the Mantel-Haenszel test).

Here we report the examination of both *MTHFR* variants in 613 CaP patients from 292 families and in 562 controls. We did not find significant differences in allele frequencies between the case and control samples, nor did we find a significant difference with respect to tumor grade and stage.

Material and Methods

Cases. Most of the families, 230 and 27 respectively, were included in a genome scan and a follow-up study (21, 22). Details of the ascertainment procedures are described in those publications. 22 families were added for an analysis of an association between *HPC2/ELAC2* polymorphisms and CaP, 8 were added for an analysis of an involvement of the *AR* in CaP (23) and 5 are new to this study.

Controls. The men were ascertained from a large sample pool that has been followed for many years as a part of a long-term CaP screening study. The subjects were screened at 6-12 month intervals with prostate specific antigen (PSA) blood test and digital rectal examination (DRE) of the prostate (24). Due to the large size of the pool, we were able to impose strict recruitment criteria. Specifically, the subjects were: a) at least 65 years old, b) never had a PSA level in excess of 2.5 ng/ml in 3 or more tests nor have they had a suspicious DRE, and c) none have a known family

history of CaP (the subjects were asked if their brother(s), father, grandfathers, maternal and paternal uncles had prostate cancer). Since we selected men unaffected with CaP who were 65 years or more, the control subjects are significantly older than the CaP subjects (25). All subjects in this study are of EuroAmerican ancestry. The Human Studies Committee of Washington University School of Medicine approved the study protocol, and informed, written consent was obtained from all participants.

MTHFR genotyping.

The C677T genotyping (dbSNP rs1801133) was done by a PCR-RFLP protocol essentially as reported (4), except that we designed new primers by Oligo 3.0 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR was performed with 1.25 uM of MTHFRe45 5'-tccctgtggtctcttcatcc-3' and MTHFRe43 5'-caaagcggaagaatgtgtca-3' primers, 1.5 mM $MgCl_2$ standard buffer and 0.7 u of *Taq* polymerase in 10 ul volume for 35 cycles. The DNA was first denatured at 94°C for 2 minutes and then amplified at 92°C for 30 sec, 55°C for 1 min and 72°C for 1 minutes, repeated 35 times. 2 ul of each reaction was electrophoresed in 2% 3:1 NuSieve agarose to determine the presence of a product. The rest of the reaction was digested overnight at 37°C with 2 u of *Hinf I* (New England Biolabs). The 677T *Hinf I* digested fragments were 150 bp and 56 bp, while the wild type (677C) was 206 bp. The DNA of the subjects was set up in 96-well tray format and the PCR, restriction digestion and gel electropheresis was performed with multi-channel pipets. Each tray included 2 control DNAs and additional 30 samples were genotyped twice. The repeated genotypes were all identical.

The A1298C SNP (dbSNP rs1801131) had a confusing restriction pattern when digested with *Mbo II* (26); therefore, we developed a template-directed dye incorporation assay for this locus (27). Briefly, 5 ng of genomic DNA was amplified with 0.125 uM of MTHFRe75 5'-gagtgggacgagttccctaa-3' and MTHFRe73 5'-tttggttctcccgagaggta-3' at 1.5 mM $MgCl_2$, standard PCR buffer. The DNA was first denatured at 94°C for 2 minutes and then amplified at 92°C for 30

sec, 55°C for 1 min and 72°C for 1 minutes, and repeated 40 times in a final volume of 7 ul. The concentration of the nucleotides was 50 uM and we used 0.25-0.5 u of Platinum *Taq* polymerase (Invitrogen, Inc.). 2 ul of each reaction was electrophoresed in 2% 3:1 NuSieve agarose to determine the presence of a 302 bp product. Unincorporated nucleotides and primers were digested by a treatment with 0.1 u of exonuclease I (USB) and 0.1 u of shrimp alkaline phosphatase (USB) at 37°C for 30 mins and then these enzymes were heat inactivated at 80°C for 15 min. To each product we added primer that abuts the polymorphic site, MTHFRe7R 5'-GAACRAAGACTTCAAAGACACTT-3', to a final concentration of 0.25 uM. The primer is made up of a mixed population at the 5th position in which 50% of the molecules have an A and the other 50% have a G. This is a silent polymorphism (26). We used the AcycloPrime-FP SNP Detection kit from Perkin-Elmer with R110-acyG & Tamra-acyT labeled derivatized Acyclo terminators. The template was first denatured at 95°C for 2 minutes and then amplified at 95°C for 15 sec and 55°C for 30 sec. The reaction proceeded for 45-60 cycles, depending on the efficiency of dye incorporation. The dye incorporation was determined on a fluorescence polarization detector, LJL Analyst™ HT (Molecular Devices, Sunnyvale, CA) in Dr. Kwok's laboratory. The nucleotide calls were determined using a macro written by B. Coleman for this purpose. Forty-two DNAs selected at random were genotyped twice and the genotypes were identical. In addition 2 controls were included on each tray to confirm genotyping accuracy.

23 cases and 36 controls were deleted either because of missing or ambiguous genotypes at either or both SNPs and because some of the controls were not of EuroAmerican ancestry. The families fell into the following categories: 23 sibships had only 1 case genotyped, 220 sibships had 2 cases genotyped, 46 sibships had 3 cases genotyped and 3 sibships had 4 cases genotyped.

Statistical and Genetic Analyses.

Maximum likelihood allele frequency estimates for the CaP cases were obtained from the USERM13 subroutine of the MENDEL computer package (28, 29). Gene frequency estimates for

the control sample were obtained by direct gene counting and used to predict Hardy-Weinberg proportions, the significance of which were evaluated with a likelihood ratio chi-square on 1 degree of freedom (30).

Haplotype frequencies were estimated using the EM algorithm as implemented in the ASSOCIATE program (31). Linkage disequilibrium estimates were converted to their "normalized" D' values, as described elsewhere (32). Equality of allele frequencies between cases and controls was assessed with a 2-sample proportion test (33). Among the unrelated cases, Mantel-Haenszel chi-squares (34) were computed to assess the relationship between the genotype and tumor grade and stage.

The evidence for linkage between CaP and each of the *MTHFR* polymorphisms was evaluated with the computer program GENEHUNTER-PLUS (35, 36).

Results

Allele frequencies of *MTHFR* in cases and controls. The SNP frequency estimates for both exons of *MTHFR* in the cases and controls are shown in Table I. Hardy-Weinberg equilibrium exists in the controls (χ^2 for the exon 4 polymorphism is 0.08 (p=0.78) and for the exon 7 polymorphism the χ^2 is 0.26 (p=0.61)).

Exon	Allele	Cases	Controls
4	C	0.6678	0.6445
	T	0.3322	0.3555
7	C	0.3139	0.3099
	A	0.6861	0.6901

Table I. Allele frequencies of MTHFR of cases and controls. Exon 4 677T and exon 7 1298C are the variant forms of the gene.

Association and linkage analysis between *MTHFR* variants and CaP. The null hypothesis that the SNP frequencies for CaP cases and controls were sampled from the same population cannot be rejected (for C677T, Z=0.48, p=0.63; for C1298A, Z=0.08, p=0.93).

We also analyzed the data with respect to Gleason score and tumor stage, when available (Table II). We drew a random sample from all sibships in which at least one brother had a Gleason score and had genotype information for exons 4 and 7. There is some sample size attrition because staging was not available on all of the subjects who otherwise met the inclusion criteria. The difference in sample size, however, is proportional to the row totals, suggesting no sampling bias. The Mantel-Haenszel chi-squares are uniformly non-significant. For the exon 4 polymorphism, χ^2=1.67 (p=0.20) and 0.58 (p=0.45) for grade and stage, respectively, while for the exon 7 polymorphism, the χ^2s are 0.78 (p=0.38) and 0.03 (p=0.86), respectively, for tumor grade and stage.

Genotype	Tumor Grade*			Tumor stage	
Exon 4	**G1**	**G2**	**G3**	**T1 + T2**	**T3 + T4**
CC	22	96	7	59	34
CT	15	110	3	72	33
TT	2	26	2	18	8
Exon 7					
AA	18	107	7	73	36
AC	15	105	4	63	32
CC	6	20	1	13	7

Table II. Distribution of *MTHFR* genotypes cross-classified by tumor grade and stage. Exon 4 677T and exon 7 1298C result in decreased enzyme activity.
*G1 = Gleason scores 2 + 3 + 4
G2 = Gleason scores 5 + 6 + 7
G3 = Gleason scores 8 + 9 + 10

Single point linkage analysis reveals no evidence of increased allele sharing among the CaP brothers in 269 sibships that contained at last 2 affected genotyped brothers. For the exon 4 polymorphism a LOD score of -0.48 and for the exon 7 polymorphism a LOD score of -0.28 was

obtained. We also analyzed a subset of the families (N=63) that have a family history of breast cancer. They had a positive LOD score for some 1p36 markers, though the highest LOD score was some distance from *MTHFR*. The LOD scores are - 0.58 for exon 4 and –0.54 for exon 7.

Linkage Disequilibrium. There is considerable linkage disequilibrium between the exon 4 and exon 7 SNPs. Estimates of the haplotype frequencies in controls are shown in Table III. The estimated frequencies differ significantly from their equilibrium expectations from ($\chi^2 = 138$, $p < 0.0001$) and give rise to a D' estimate that is 90.5% of its maximum value.

Position		Expected*	Estimated
677	1298	Frequency	Frequency
C	C	0.200	0.299
C	A	0.445	0.345
T	C	0.110	0.010
T	A	0.245	0.345

Table III. Expected and estimated haplotype frequencies of 2 variants in *MTHFR* (*under the hypothesis of no allelic association).

Discussion

The *MTHFR* maps to 1p36, at or close to the chromosomal region that has been implicated, by linkage analysis (21, 37), to harbor a predisposition gene for familial CaP associated with other tumors. The LOD_{max} in the latter report was with a marker in 1p35 (21). The highest LOD scores of these 2 studies are separated by ~23 cM (38), or ~15 Mb, on the December 2001 genome assembly, (http://genome.ucsc.edu/cgi-bin/hgGateway?db=hg10), with *MTHFR* ~ 3 Mb distal to the Gibbs et al. signal and 18 Mb distal to the Suarez et al. signal. Two variants of *MTHFR* at amino acids 222 and 429 respectively, affect the levels of 5-methylTHF, which in turn affects DNA methylation and DNA synthesis. However, the effect is weak and may be ameliorated by the presence of dietary folate and vitamin B12. Alternations in DNA methylation have been observed

in urological malignancies [e.g. (18)], including hypermethylation of promoter regions and genome-wide hypomethylation. Kimura et al., reported results suggesting that the 677T (valine) was more frequent among CaP patients and tended to be associated with higher-grade tumors (20). In our study, neither result could be replicated. We did not find evidence that *MTHFR* is involved in familial prostate cancer etiology. However, among the confounding variables of the MTHFR mutants' effects are the dietary effects of alcohol, folate and methionine intake (39), and we do not have information on the dietary habits of our patients or controls.

Isotalo and colleagues, in a study of *MTHFR* of 119 neonates and 161 fetal samples, did not find triple or quadruple variant combinations in the neonates, though they were present in the fetal samples ((40). The authors suggested that the triple and quadruple variant combinations may decrease the viability of fetuses and are of a possible selection disadvantage to fetuses with increased number of variant *MTHFR* alleles. Rosenberg et al., did not observe the doubly variant 677TT/1298CC combination in unselected 188 white Israelis, 82 Japanese and 174 Ghanaian Africans (41). These authors also reported that the 677T allele arose on a chromosome that had adenosine at position 1298; therefore, one can infer that the 1298C variant of *MTHFR* must have been independent event to the 677 mutation, and it arose on the 677C background. Two other groups also did not observe doubly homozygous individuals in their studies of 525 and 353 individuals, respectively, (42), (43). They suggest that there is a selection against these individuals. However, we found 2 doubly variant individuals (677TT/1298CC) and 7 triple variant individuals in the adult male EuroAmerican population we genotyped. Therefore, we can confirm that recombination occurs in the 2.1 kb of DNA that separates these two polymorphic sites, but we cannot provide evidence that *cis* combination of mutations completely compromises fetal viability.

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